Natural Product Sciences
19(1) : 49-53 (2013)

Antioxidant Activity and Its Mechanism of *Paeonia lactiflora* Pall Extract

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Abstract – *Paeonia lactiflora* Pall (PL) has been used as a traditional herbal medicine in China, Korea, and Japan for more 1,200 years. PL has reported to have antioxidant activity and protective effect of cells from oxidative stress, although the mechanism has not been verified. FOXO3a is a transcription factor that binds to its target gene's consensus FOXO binding site. FOXO3a protein modulates the various biological functions including cell cycle control, apoptosis, DNA repair, and ROS detoxification. Therefore, FOXO3a activity is associated with cancer, aging, diabetes, infertility, neurodegeneration, and immune system dysfunction. Here we found that FOXO3a was activated by PL extract. Transcriptional target genes such as MnSOD, p27, and GADD45 were activated by PL extract. Protein levels of MnSOD and catalase were increased, consequently, ROS level was reduced in HEF cells by PL extract. These findings suggest that PL extract has an antioxidant activity through FOXO activation and thereby activation of FOXO target genes, MnSOD and catalase.
Keywords – FOXO3a, Antioxidant, *Paeonia lactiflora* Pall.

Introduction

Paeonia lactiflora Pall (PL, Paeoniaceae) has been used as a medicinal herb in China, Korea, and Japan for more 1,200 years. The extract of PL is known as total glucoside of peony (TGP) that contains paeoniflorin, albiflorin, oxypaeoniflorin, benzoylpaeoniflorin, oxybenzoylpaeoniflorin, paeoniflorigenone, lactiflorin, galloylpaeoniflorin, paeonin, paeonolide, and paeonol (He and Dai, 2011). Among them, paeoniflorin is the most abundant (> 90%) of TGP. PL has an analgesic activity (Lee *et al.*, 2008a), anti-inflammatory activity (Cao *et al.*, 2011; Kim and Ha, 2009), immunomodulatory activity (Tsuboi *et al.*, 2004; Wu *et al.*, 2007), antioxidant activity (Lee *et al.*, 2005), and protective effects of cells from oxidative stress (Lee *et al.*, 2008b).

FOXO3a is transcription factor that belong to the forkhead box O (FOXO) class. FOXO consist of four members: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX), and FOXO6. FOXO has a highly conserved DNA-binding domain which referred to as a winged-helix domain. FOXO protein functions as transcriptional activators by binding to the consensus binding sequencing TTGTTTAC (Furuyama *et al.*, 2000). FOXO3a proteins can modulate the various biological functions such as cell cycle control, apoptosis, DNA repair, and oxidative stress through regulation of its target genes (Huang and Tindall, 2007). FOXO3a can promote cell cycle arrest by transcriptional regulation of p27, cell cycle inhibitor. FOXO3a can also regulate apoptosis through transcriptional regulation of Bim, proapoptotic factor. FOXO3a protects cells from oxidative stress by directly transcriptional regulation of manganese superoxide dismutase (MnSOD) and catalase. FOXO3a activates DNA repairs of damaged DNA by transcriptional regulation of GADD45. FOXO3a activity is reported to be associated with cancer, aging, diabetes, infertility, neurodegeneration, and immune system dysfunction (Maiese *et al.*, 2008).

In this report we showed that FOXO3a was activated by PL extract. Protein level of FOXO3a was increased. And activities of target genes of FOXO3a were increased by PL extract. Reactive oxygen species (ROS) level was also reduced by PL extract in HEF cells through activation of FOXO target genes, MnSOD and catalase.

Experimental

Cell culture – A primary human embryonic fibroblast

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(HEF) cells were established from an abortus at Hallym University Hospital (Kim *et al.*, 2005). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Bio-Whitaker, Basel, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO_2.

Preparation of the crude extract of PL – PL was purchased from Dae Kwang Herb Medicine Co., Ltd., Chuncheon, Korea and the voucher specimen (No. RIC-1434) was deposited at the center for efficacy assessment and development of functional foods and drugs (Regional Innovation Center), Hallym University, Republic of Korea. Roots of PL (500 g) mixed with 3 liter of 70% ethanol-water solution in a 5,000 mL round bottom flask fitted with a cooling condenser which was used to perform the extraction. The extraction temperature was controlled at 70 °C with a water bath to allow ethanol boiled continuously. Extraction was carried out for 6 h. The extracts were combined and concentrated under reduced pressure with a Model EYELA N-1000 rotary evaporator (Tokyo Rikakikai, Tokyo, Japan), which yielded 91.5 g (18.3%) of the crude extract for biological test.

Western blot analysis – Cells were harvested and lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% NP-40 containing a mixture of protease inhibitors). 40 μg of protein was separated on SDS-polyacrylamide gel and then transferred to immune-blot PVDF membrane. The membranes were incubated with primary antibodies. The membranes were then incubated with the secondary antibody conjugated with anti-mouse or anti-rabbit IgG-horseradish peroxidases. Protein detection was performed using the ECL system (Amersharm Biosciences, New Jersey, USA). Anti-FOXO3a and anti-phospho-FOXO3a (T32) antibodies were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-catalase antibody was purchased from Novous. Anti-MnSOD antibody was purchased from BD Pharmingen (New Jersey, USA). Anti-actin antibody was purchased from Sigma. Horseradish peroxidase-coupled anti-rabbit and anti-mouse were from Pierce Technology Corporate (Rorkford, USA).

DNA construct and transfection – FHRE-Luc vector was purchased from Addgene (Plasmid #1789). FHRE-Luc vector has three FOXO3a response elements. Cells were transfected with plasmid DNA using Lipofectamine LTX (Invitrogen, Califonia, USA).

Luciferase assay – HEF cells were seeded in 24-well plate at 24 h before transfection. HEF cells were transfected with plasmid DNAs. pCMV-β-gal (CMV promoter-*E.coli* beta gealactosidase gene) was co-transfected for calibration of transfection efficiency. After 24 h, cells were treated with PL extract for 24 h.

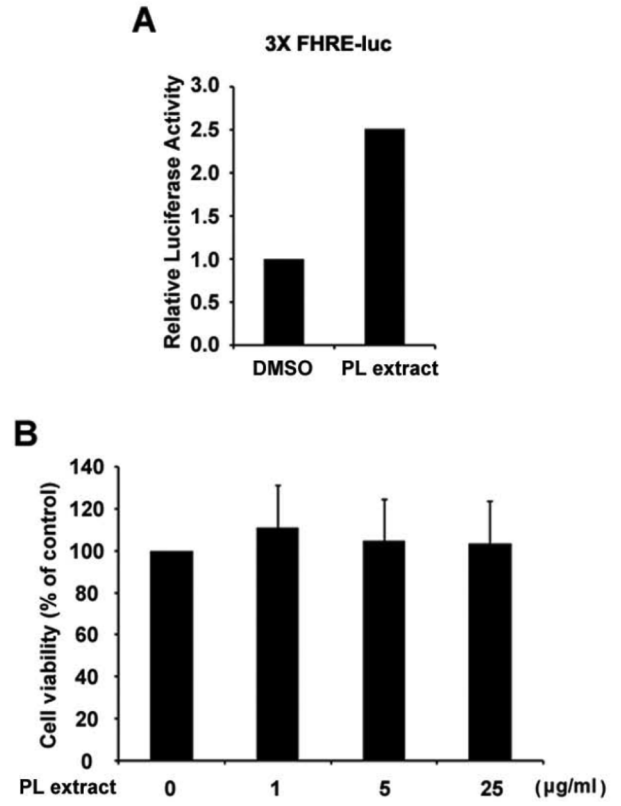

Fig. 1. FOXO3a promoter activity and cell survival in response to PL extract.

(A) HEF cells were transfected with FHRE-Luc DNA. After 24 h, PL extracts were treated to the transfected cells for 24 h. Cells were lysed and luciferase activities were measured. To calibrate the transfection efficiency, pCMV-β-gal was cotransfected and β-galactosidase activity was measured. (B) HEF cells were treated with PL extract (1, 5, and 25 μg/mL) for 24 h. MTT solution was incubated for 4 h, the absorbance of each well was measured at 570 nm.

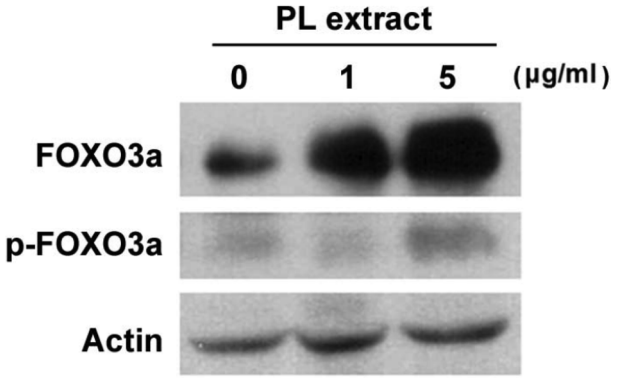

Fig. 2. Change of FOXO3a protein level in response to PL extract. HEF cells were treated with PL extract for 24 h. The protein levels of FOXO3a, p-FOXO3a, and actin were measured by western blot analysis.

Luciferase and β-galactosidase activities in the extract of the transfected cells were measured using a procedure and reagents provided by Promega (Fitcgburg, USA) according to the manufacturer's instructions.

MTT assay – 5×10^3 HEF cells were seeded in 24-well plate and treated with PL extract for 24 h. MTT solution was incubated for 4 h, the absorbance of each well was measured at 570 nm.

Measurement of reactive oxygen species – Cells were incubated with 30 μM DCDHF-DA for 30 min at 37 °C. Cells were washed twice with PBS, and detached with trypsin. Cells were sonicated and centrifuged (12,000 g, 20 min, 4 °C). Fluorescence intensity was measured in the supernatants using a fluorescence spectrometer (SpectramaxM2, Molecular Devices, USA) (λ_{ex}: 485 nm; λ_{em}: 530 nm). Fluorescence intensities were normalized by total amount of proteins (Alaimo *et al.*, 2011).

Results and Discussion

To investigate whether PL extract activates FOXO3a, HEF cells were transfected with FHRE-Luc vector containing three FOXO3a response elements. After 24h, cells were treated with PL extract for 24 h and then luciferase activity was measured for cell extract. Luciferase activity was increased by about 2.5 folds in response to PL extract (Fig. 1A). To test the cytotoxic effect of PL extract, HEF cells were treated with PL extract (1, 5, and 25 μg/mL) for 24 h and then cell viability was measured by MTT assay. PL extract did not affect the cell viability in these doses (Fig. 1B).

Because PL extract activates FOXO3a, the protein level of FOXO3a was assessed by western blot analysis. HEF cells were treated with PL extract for 24 h. The results show that the protein level of FOXO3a was increased

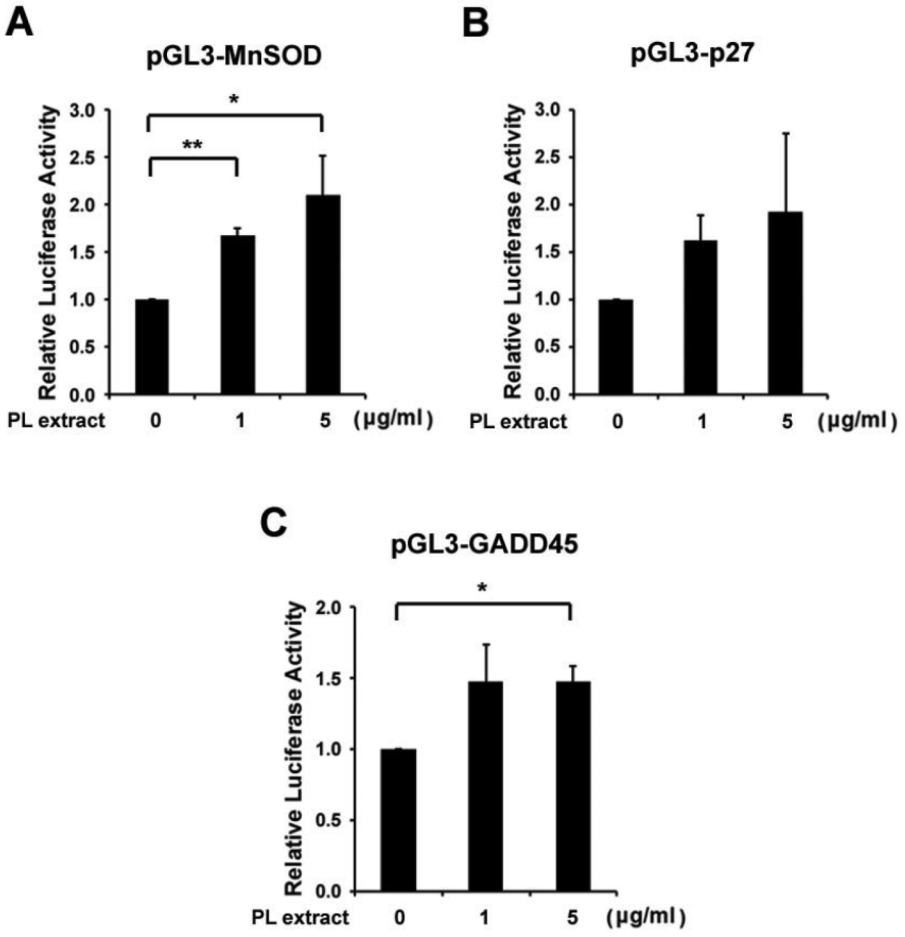

Fig. 3. Change in promoter activities of FOXO3a target genes in response to PL extract. HEF cells were transfected with pGL3-MnSOD (A), pGL3-p27 (B), and pGL3-GADD45 (C), respectively. After 24 h, cells were treated with PL extract for 24 h and luciferase activities were measured. To calibrate the transfection efficiency, pCMV-β-gal was cotransfected and resulting β-galactosidase activity was measured. The error bars indicate standard deviations of results obtained from three independent experiments and the statistical significance is indicated as *$p < 0.05$ and **$p < 0.01$ by Student's t-test.

more than 5 times in response to PL extract but phosphorylation level of FOXO3a (Thr 32) was not changed (Fig. 2). FOXO3a was regulated by posttranslational modifications such as phosphorylation, acetylation, and ubiquitination. Stability, DNA binding activity, and cellular localization of FOXO3a were affected by these posttranslational modifications. The serine-threonine kinase AKT/protein kinase B (PKB) phosphorylates FOXO3a. These phosphorylation leads to nuclear exclusion of FOXO3a and inhibits FOXO3a activity. Inhibition of AKT increases the nuclear translocation of FOXO3a and its target gene activation (Brunet *et al.*, 1999). FOXO3a was directly phosphorylated by ERK and degraded via an MDM2-mediated ubiquitin-proteasome pathway (Yang *et al.*, 2008). AMP-activated protein kinase (AMPK) phosphorylates FOXO3a at Thr 179, Ser 399, Ser 413, Ser355, Ser 588, and Ser 626, and activates FOXO3a without affecting subcellular localization (Greer *et al.*, 2007). FOXO3a was phosphorylated by IkappaB kinase (IkK), a regulator of NF-κB. Activities of FOXO3a were inhibited by this phosphorylation (Hu *et al.*, 2004). SIRT1, an NAD-dependent class III HDAC, interacts with FOXO3a in response to oxidative stress and deacetylates FOXO3a. Deacetylated FOXO3a induces cell cycle arrest but inhibits cell death (Brunet *et al.*, 2004). It is still unknown how FOXO3a is activated by PL extract.

To investigate whether the target genes of FOXO3a such as MnSOD, p27, and GADD45 were activated by PL extract, luciferase assay was performed. HEF cells were transfected with pGL3-MnSOD, pGL3-p27, and pGL3-GADD45 reporter plasmid DNA, respectively. After 24 h, cells were treated with PL extract for 24 h and then luciferase activities were measured. Promoter activity of MnSOD, p27, and GADD45 was increased by PL extract with dose-dependent manner (Fig. 3).

It was reported that PL has an antioxidant activity. The ethanol extracts of the peony root and its major active components including gallic acid and methyl gallate exhibited antigenotoxic and antioxidant activity by scavenging free radicals and protecting against oxidative DNA damage, although the mechanism has not been verified (Lee *et al.*, 2005). PL is also known to have a protective activity against H_2O_2-induced apoptosis although the mechanism was not verified. When PC12 cells were treated with 0.5 mM H_2O_2 for 2 h, cell viability was 45.3% compared to the control. However, when cells were pretreated with PL extract, cell viability was about 75% (Lee *et al.*, 2008b). To investigate the molecular mechanism of antioxidant activity of PL extract, the protein levels of MnSOD and catalase, FOXO3a target

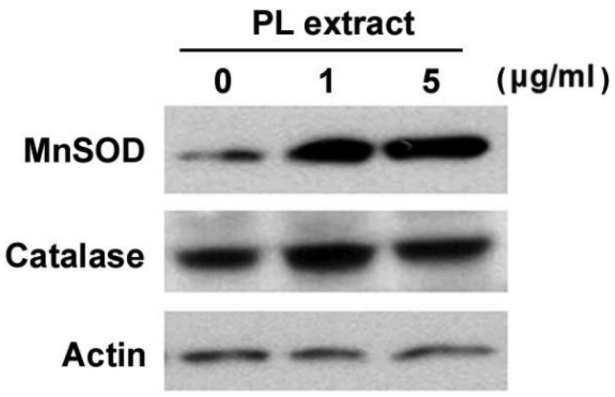

Fig. 4. Change in protein levels of MnSOD and catalase in response to PL extract. HEF cells were treated with PL extract for 24 h. The protein level of MnSOD and catalase were measured by western blot analysis.

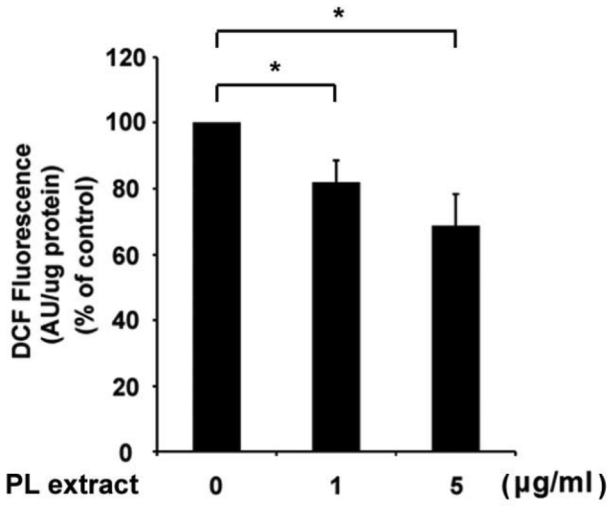

Fig. 5. Change of ROS level in response to PL extract. HEF cells were treated with PL extract for 24 h. Cells were incubated with 30 μM DCDHF-DA for 30 min at 37 °C. Cells were washed and detached with trypsin. Cells were sonicated and centrifuged. ROS measured by fluorescence spectrometer (λ_{ex}: 485 nm; λ_{em}: 530 nm). Fluorescence intensities were normalized by total amount of proteins. The error bars indicate standard deviations of results obtained from five independent experiments and the statistical significance is indicated as *$p < 0.05$ by Student's t-test.

genes, were measured by western blot analysis. HEF cells were treated with PL extract for 24 h. The results show that MnSOD and catalase protein level was increased by PL extract (Fig. 4). Thereby ROS level was decreased by PL extract in HEF (Fig. 5). Although ROS have crucial role in various biological processes, excess ROS level causes damage of nucleic acids, proteins, and lipids. It results in eventually various diseases such as cancer, diabetes, neurodegeneration, atherosclerosis, and aging (Ray *et al.*, 2012). In this study we showed that PL extract

activated FOXO3a to promote the detoxification of ROS by transcriptional activation of its target genes, MnSOD and catalase in HEF cells.

Acknowledgements

This work was supported partly by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A6A1048184).

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Received February 20, 2013
Revised March 7, 2013
Accepted March 14, 2013